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Studies of Second Structure of 16S rRNA Gene in Uncultured *Paenibacillus* Species Clone T-168

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ABSTRACT

The 16S rRNA gene was sequenced and deposited in GenBank, Accession number: AB828175.1. The result of sequencing was compared by DNAMAN software. RNA structure analysis software was used for prediction and analysis of secondary structures of the 16S rRNA gene in three species of Uncultured *Paenibacillus*, including, clone T-168, clone SGR-280 and clone SGR-10. It was designed to make algorithms accessible for a variety of user needs. These include both free energy and change parameters at 37°C and enthalpy change parameters to allow prediction of conformation stability at an arbitrary temperature. The results for the minimum free energy (MFE) prediction in form of the secondary structure in dot bracket notation and the free energy and centroid secondary structure were revealed between sequences. The minimum free energy prediction was -205.00, -562.20, and -574.60 kcal/mol for clone T-168, clone SGR-280, and clone SGR-10 respectively. Furthermore, the ensemble diversity is 183.09, 380.21 and 284.80 for clone T-168, clone SGR-280 and clone SGR-10, respectively. The centroid secondary structure in dot-bracket notation with a minimum free energy of -153.20, -449.92 and -503.92 kcal/mol for clone T-168, clone SGR-280 and clone SGR-10 were reported respectively. According to these results, high variation between clone T-168, clone SGR-280 and clone SGR-10 were found, but there were high homology between both clone SGR-280 and clone SGR-10.

Keywords: Uncultured *Paenibacillus* clone T-168, Secondary structure 16S rRNA gene

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INTRODUCTION

The 16S rRNA gene in Uncultured *Paenibacillus* species were studied by researches regarding phylogenetic of these species. Generally, there was not a high variation between 16S rRNA genes in organisms, but in some organisms, comparing 16S rRNA genes between them revealed some variations. The 16S rRNA gene is important for finding the descent of species. In this study, we aimed for finding the rate of homology between species of the *Paenibacillus* when compared by secondary structure of the 16S rRNA gene. Our understanding of the role of ribosomal RNA in translation has been in a state of rapid evolution in recent years. No longer considered a mere scaffold for the positioning of ribosomal proteins, rRNA currently appears instead to play an active and probably crucial part in protein synthesis (reviewed in Noller, 1984). The 16S region has been studied on the presence of functional motifs (Berg *et al.* 1989; Nodwell and Greenblatt, 1993; Pfeiffer and Hartmann, 1997) and secondary structures (Nour, 1998; Liiv *et al.* 1998). The region of 16S rRNA was revealed in prokaryotic diversity at different species (Gurtler and Stanisich, 1996; Garcia-Martinez *et al.* 1999). In recent study, 16S rRNA genes from some microbial species were analyzed with helping of PCR techniques when restricted by endonuclease digestions (Papke *et al.* 2004; Pernthaler and Pernthaler, 2005; Philippe, 1993). These results also were supported on at least five phyletic lines especially *Panibacilus* (Ash *et al.* 1993). A very small variation between sequences of 16S rRNA genes was observed which could be used to classify and identify organisms correctly. Very small differences in sequences from one bacterial species to another can be used to classify and identify organisms correctly (Avaniss-Aghjani, 1994). The PCR primers were designed against *Paenibacillus* species. It was found to have seven nucleotide differences compared to *P. alvei* and 10 nucleotide differences compared to *P. polymyxa*. These results confirmed against the 16S rRNA gene in the Bacteria. However, the rate of homology in 16S rRNA genes, when compared between sequences, is very high, but on the secondary and tertiary structures of rRNA, there may has been a different situation about RNA genomics of Bacteria. The shape of secondary structures can be predicted by free energy minimization with nearest neighbor parameters to evaluate stability (Mathews *et al.* 1999; Stajich, *et al.* 2002; Zuker and Stiegler, 1981; Zuker, 1989; Zuker, and Sankoff, 1984; Gaspin and Westhof, 1995; Chen and Dill, 2000; Wuchty *et al.* 1999; Ding and Lawrence, 2003).

A predicted secondary structure can guide further experiments or comparative sequence analysis (Wolfinger *et al.* 2004) and aid in the design of RNA molecules (Diamond *et al.* 2001; Pappalardo *et al.* 1998). The first step for understanding the rRNA function is to determine its structure (Mathews and Turner, 2006). Secondary structure of RNA revealed as a set of nucleotides (AU, GC and GU) can be compared between sequences (Gutell *et al.* 2002; Pace, 1999). The analysis of sequences will be determined where they are conserved in multiple sequences and instances of compensating base pair changes occur. The variation of base pair is an indicator for comparative sequences. As an alternative to comparative analysis, the secondary structure of an RNA can be predicted for a single sequence using thermodynamics (Mathews and Turner, 2006). The thermodynamic methods are based on nearest neighbor rules that predict the stability of a structure as quantified by folding free energy change (Xia *et al.* 1998; Mathews *et al.* 1999; Mathews *et al.* 2004; Evers and Giegerich, 1999). Another

alternative method for structure prediction is the prediction of a structure with the highest sum of pairing probabilities, called the maximum expected accuracy structure (Lu *et al.* 2009; Hamada, 2009). In this study we aimed to discuss about the relationship between secondary structure of RNA by using thermodynamics analysis between sequences of rRNA in *Paenibacillus* species.

MATERIALS AND METHODS

The culture samples

The *Paenibacillus* samples were collected from *Salmo trutta caspius* and *Salmo trutta fario* that were caught from Tonekabon River-Iran. The specimens representing fragments of fins were fixed in 96% ethanol. Total DNA was extracted from fin tissue using the method described by Taggart *et al.* (1992), with minor modifications. Briefly, tissue was digested in a digestion buffer, containing; (100 mM NaCl, 10 mM Tris pH 8.0, 25 mM EDTA PH 8.0, 0.5% sodium dodecyl sulphate (SDS), 0.1 mg/ml proteinase K) for 18 hours at 50°C. Samples were then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (24:25:1), then samples were centrifuged for 5 min in room temperature, again ethanol(96%) was added for washing the DNA, and finally DNA was solved in 50µl TE buffer. Samples were stored at -20°C until using for PCR reaction.

Primers for amplification of 16S rRNA gene

The sequences of 16S rRNA genes were deposited in GenBank, so we designed primers of a 16S rRNA gene that was reported in GenBank. The universal primers could be amplifying around 1400 base pairs in the full length of the gene. The primers include:

Forward Primer: 5' CACCTCCCTTACACCGAGAA 3'

Reverse Primer 5' GCCGAGTTCCTTCTCTTCCT 3'

The PCR amplification

The PCR reaction used 10µg PCR reactions contained: 1µl template DNA, 2 µl forward primer (100 ng/µl), 2 µl reverse primer (100 ng/µl), 2 µl dNTP mix (2.5mM each), 5 µl 10X buffer, 0.5 µl Taq enzyme (3U/µl), Water 37.5 µl, in a total volume of 50µl. The cycles were 1 cycle of 95°C for 10 min, 30 cycles of 94°C for 30 Sec., 52.3°C 30 Sec., and 72°C, 90 Sec. Two to ten µl of each PCR reaction were run on 1.5% agarose gel in TAE buffer containing ethidium bromide. One µl 500bp DNA ladder (Gibco-BRL) was used as a size standard. Then DNA full length DNA was visualized by ethidium bromide and photos were taken by Gel DOC, Bio RAD Company.

Nucleotide sequencing

The PCR products were separated by electrophoresis in a TAE agarose gel containing ethidium bromide using standard protocols. The desired PCR product band was excised using a clean, sterile razor blade or scalpel (band was visualized in a medium or long wavelength (e.g., ≥ 300 nm) UV light, and excised quickly to minimize exposure of the DNA to UV light). The minimum agarose slice was transferred to a 1.5 ml micro centrifuge or screw cap tube and then purified by Gel Extraction Spin-50 (RKT33) kit, Chromous Geni-India. Quantification was done by loading all of eluted sample in 1% agarose gel and comparing with standard molecular marker (500 bp DNA ladder). Only samples with good concentration (>50 ng/ μ l) were selected and subjected to sequencing.

Sequence data analyses

Sequenced data were analyzed mostly by DNAMAN and RNA fold web server genetics program.

Database search

The database search of sequences for a possible match to the RNA sequence of 16S rRNA genes was conducted using the BLAST algorithm available at the National Centre for Biotechnology, Accession number: AB828175.

RESULTS

In this study, we extracted DNA from Uncultured *Paenibacillus* clone T-168. The genomic DNA was run on the gel electrophoresis and is shown in Figure 1. Also, one pair of primers that could be amplified around 700 base pairs was designed (Figure 2). The universal primers were selected from sequence of 16S rRNAs of some species of the *Paenibacillus*. However, we designed the primers for full length but we only sequenced from the forward primer. The forward primer could amplify 677 base pairs (Figure 3).

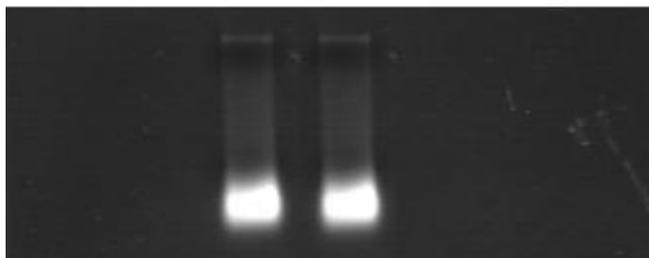


Fig. 1: Total genomic DNA of Uncultured *Paenibacillus* species clone: T-168.

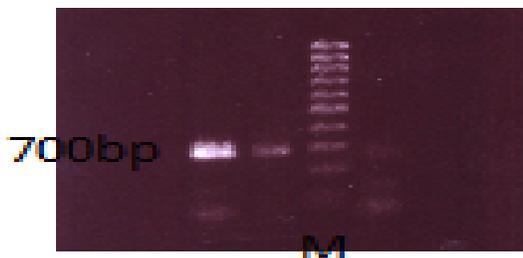


Fig. 2: PCR product of partial sequence of the 16S rRNA gene in UP clone: T-168. M: Size marker 1000bp.

SEQ AB828175: 677 bp;
 Composition 206 A; 156 C; 164 G; 151 T
 Percentage: 30.4% A; 23.0% C; 24.2% G; 22.3% T
 Molecular Weight (kDa): ssDNA: 209.42 dsDNA: 417.4

ORIGIN

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1  TCATCTCTTC TTCACTCGG AGAGACGTGA TGTGCAGCAT CTATATTCAT CTCTACCAGG
61  TTGCCAGGGT GTAGCACAAG AACCATTCCC CCAGCACAGT TCTTCAGCAA TACTTATACT
121  GTAAGGCAAT TGGCAGGGGA CGACCTCAGG CAGATTATTA TCTGTGATGA TAGGGAAGAG
181  GCGGAAAAAG TTTTACCCTT CTGGTTTTCA AACTAACCCC AACAAAGTGG GGAGGGGTTC
241  GATTACTCAG AGAACAAATG TCTTCTGTCT GAATGGAAAA AACAGGTCGT GGACAATTGG
301  GGGAGCTCCC AGGACATGAA CCACACCTTT ACGTTCGTTG CCCAAATGGA AGAAGCCCCC
361  CCCCAGGAAT TTCACTGAA AAAAAAGGGC GGGGTGGGGA AGACCTCAA TCAAAATACC
421  CCTTTTACT TGGGCTGCAC ACTTCTTACA GTGGATAGGC CGACGTCAAG CGAAGCCGAG
481  AGGCGGGGTG AATCCATAAA AATGAGTGTG AGCCGGCGGC GCATAATGCA ACTCGCCTGG
541  AAGAAGTCGA AATTGTTTTT AATTCTACAT CAAGGTGCC AAATGGGGGC ATTAACGGG
601  TTTTTTAACC CCCCTAAAAG GGATAAAAAA AAAGAAAAAA AAATAAAATA GGCCCTTCTC
661  TCTCAGGAGG CCCCCC
  
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Fig. 3: The sequence of the 16S rRNA gene that provided with DNAMAN Software.

Comparing of the 16s rRNA gene in uncultured *Paenibacillus* species clone T-168, Uncultured *Paenibacillus* sp. clone SGR10 and Uncultured *Paenibacillus* sp. clone SGR280 by DNAMAN software, version 6.0

In the present study we analyzed three sequences that reported in GenBank. The result of sequencing when compared with DNAMAN software showed almost medium homology(72%) between 16S rRNA genes in uncultured *Paenibacillus* clone T-168 , clone SGR10 and clone SGR280, however between both sequences of clone SGR10 and clone SGR280 high homology was reported (Figure 4).

RNA structure analysis software

RNA structure analysis software was used for prediction and analysis of secondary structures. It was designed to make algorithms accessible for a variety of user's needs. The algorithms implemented in RNA structure use nearest neighbor parameters to predict the

stability of secondary structures. These include both free energy and change parameters at 37°C and enthalpy change parameters to allow prediction of conformation stability at an arbitrary temperature. For analysis of prediction secondary structure of 16S rRNA we used three species of *Paenibacillus*, including, uncultured *Paenibacillus* species clone T-168, uncultured *Paenibacillus* species clone SGR10 and uncultured *Paenibacillus* species clone SGR280. First, we provided results for the minimum free energy (MFE) prediction in form of the secondary structure in dot bracket notation and the free energy and centroid secondary structure were revealed in uncultured *Paenibacillus* clone T-168, clone SGR10 and clone SGR280 for minimum free energy prediction -205.00 kcal/mol, -562.20 kcal/mol and -574.60 kcal/mol respectively which are given in figures 4a-c and 5a-c. The results of the free energy of thermodynamic ensemble prediction in uncultured *Paenibacillus* clone T-168, clone SGR10 and clone SGR280 are -217.63 kcal/mol, -594.49 kcal/mol and -582.63 kcal/mol, respectively. The results of the frequency of the MFE structure in the ensemble of three species cited in above was 0.00 %. Furthermore, the ensemble diversity is 183.09 for uncultured *Paenibacillus* clone T-168, the ensemble diversity is 380.21 for uncultured *Paenibacillus* clone SGR 280 and the ensemble diversity is 284.80 for uncultured *Paenibacillus* clone SGR10. The centroid secondary structure in dot-bracket notation with a minimum free energy of -153.20 kcal/mol is given for uncultured *Paenibacillus* clone T-168, and -449.92 kcal/mol and -503.92 kcal/mol were reported for clone SGR280 and clone SGR 10, respectively (Figure 6a-c).

Figures 7a-c present the mountain plot representation of the MFE structure, the thermodynamic ensemble of RNA structures, and the centroid structure. Additionally, we present the positional entropy for each position. Figures 8a-c include dot plots containing base pair probabilities. There was a variation between uncultured *Paenibacillus* clone T-168, clone SGR10, and clone SGR280. However, a high homology was observed between both clone SGR10 and clone SGR280.

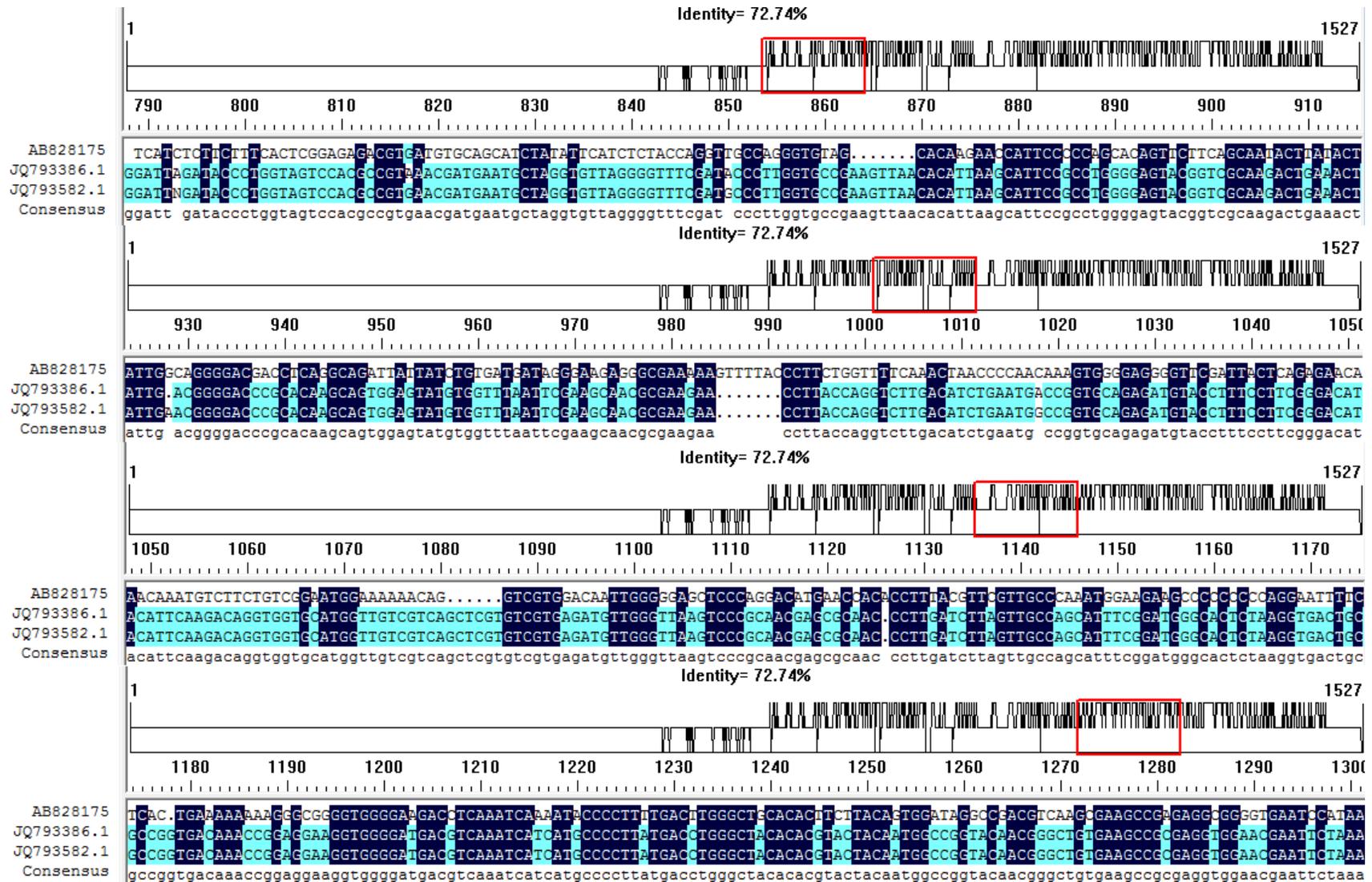


Fig. 4: 16S rRNA genes in uncultured *Paenibacillus* clone T-168(GenBank: AB828175), clone SGR 10 (GenBank: JQ793386.1) and clone SGR 280 (GenBank: JQ793582.1) were analysed by DNAMAN software. The results are showed a medium homology between clone T-168, clone SGR 10 and clone SGR 280 (72%) but a high homology was observed between clone SGR 10 and clone SGR 280.

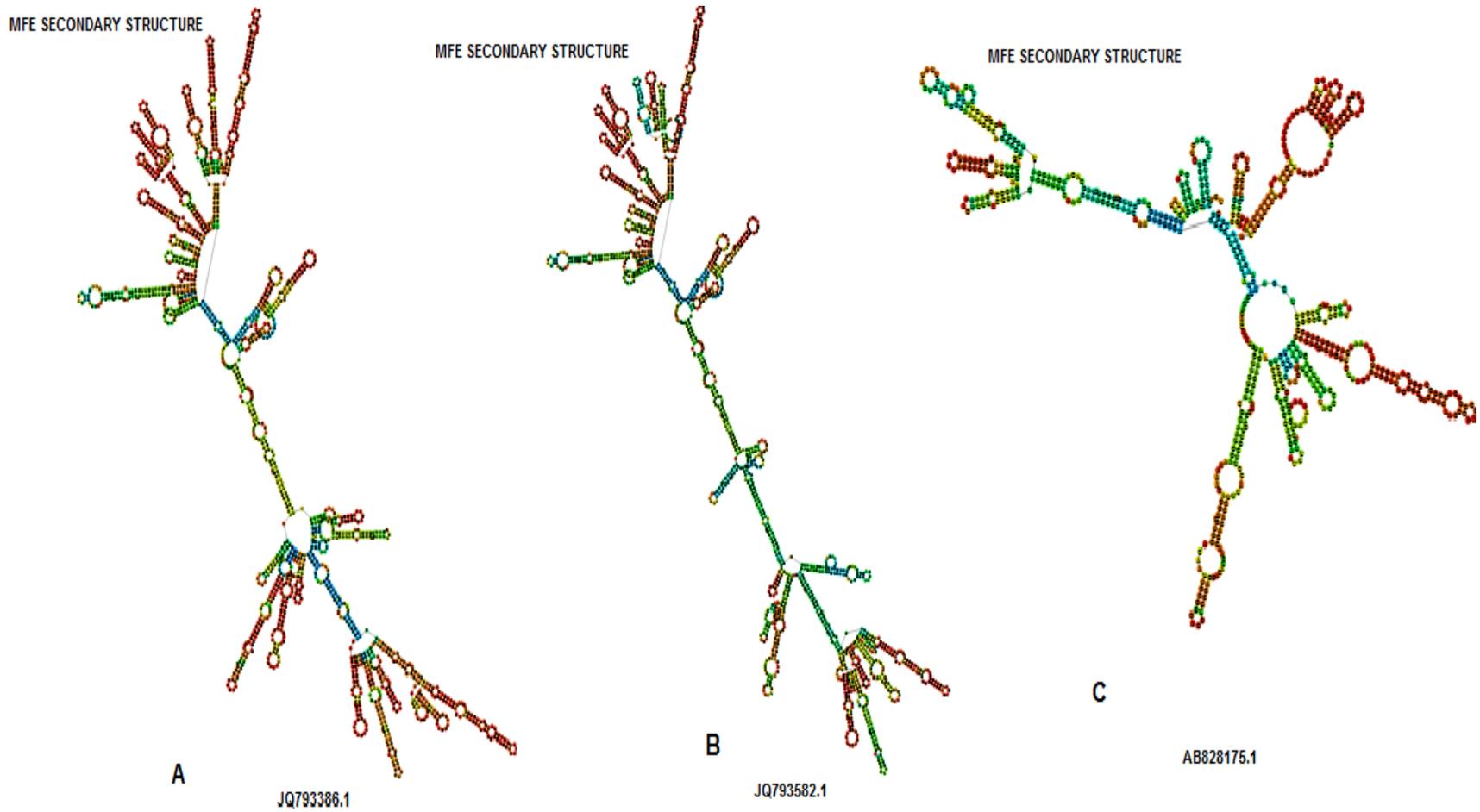


Fig. 5a-c: Screenshots of a MFE secondary structure of rRNA in uncultured *Paenibacillus* clone SGR-10, GenBank: JQ793386.1 (A), clone SGR 280, GenBank: JQ793582.1 (B) and clone T-168, GenBank: AB828175(C). We display the barrier tree and provide the transition rates matrix for download.

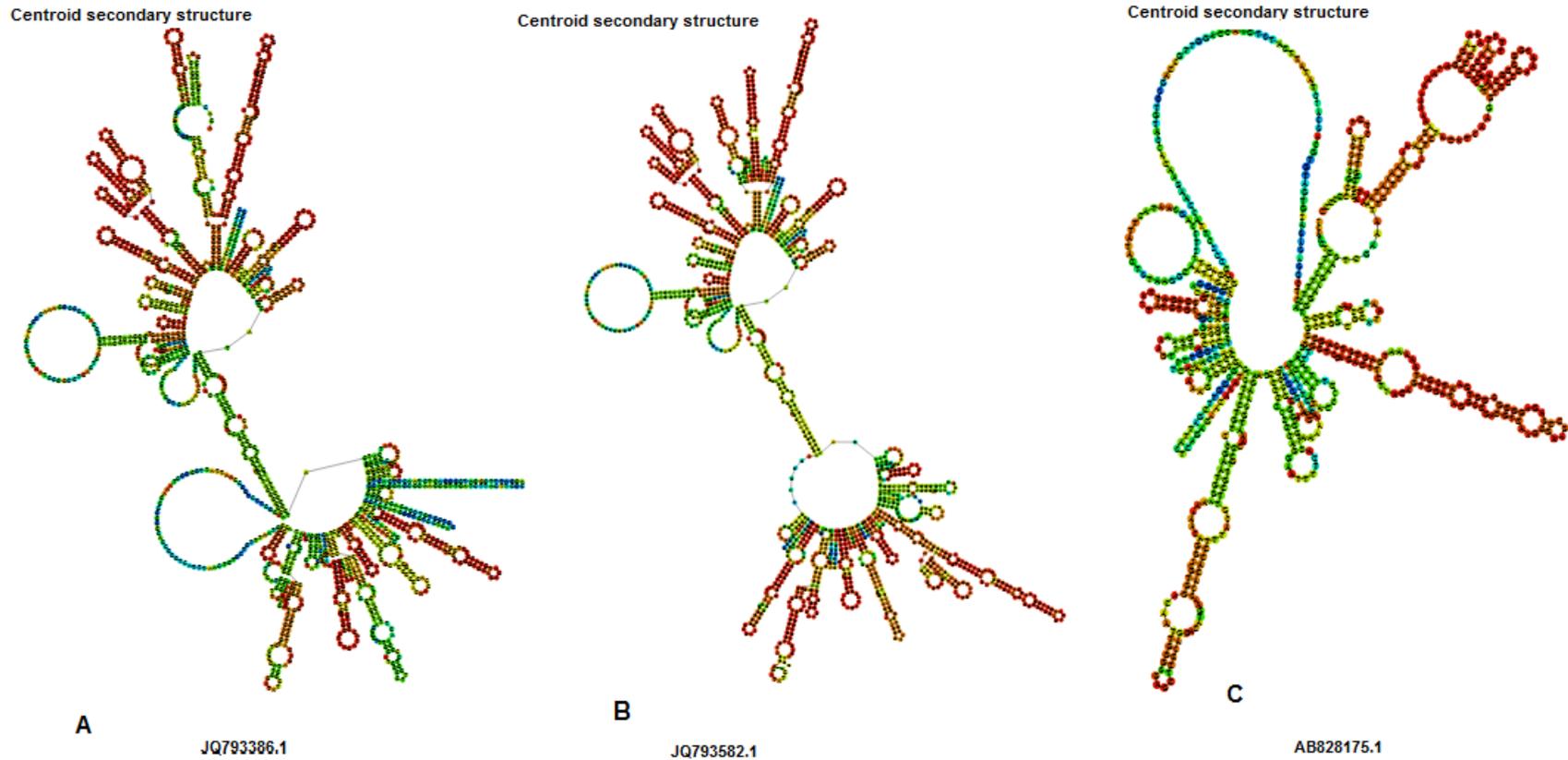


Fig. 6a-c: Screenshot of a secondary structure of rRNA in uncultured *Paenibacillus* clone SGR-10, GenBank: JQ793386.1 (A), clone SGR 280, GenBank: JQ793582.1 (B) and clone T-168, GenBank: AB828175(C). We display the barrier tree and provide the transition rates matrix for download.

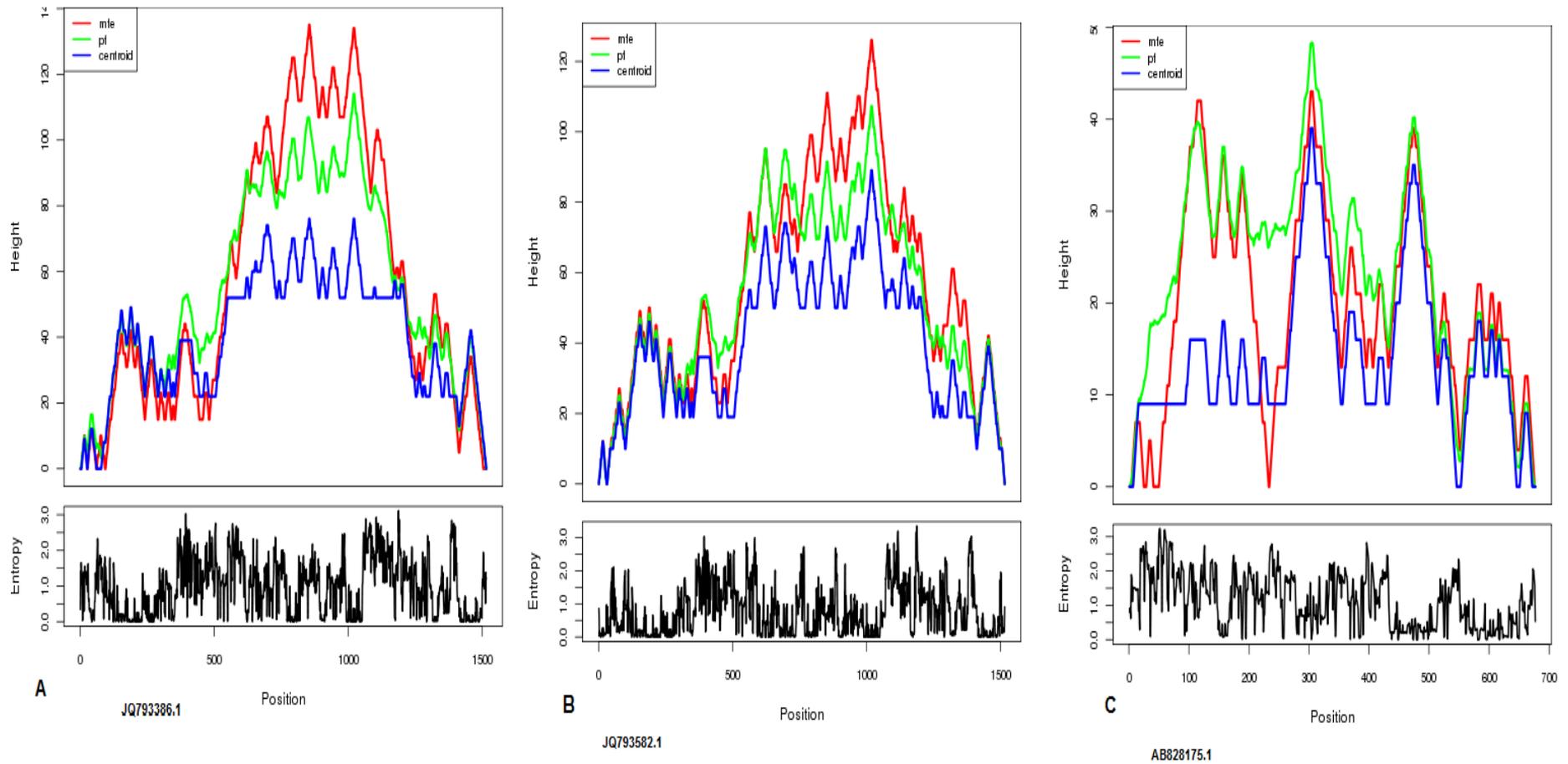


Fig. 7a-c: Mountain plot representation of the MFE structure, the thermodynamic ensemble of RNA structures, and the centroid structure. Additionally we present the positional entropy for each position. Screenshot of a secondary structure of rRNA in uncultured *Paenibacillus* clone SGR-10, GenBank: JQ793386.1 (A), clone SGR 280, GenBank: JQ793582.1 (B) and clone T-168, GenBank: AB828175(C).

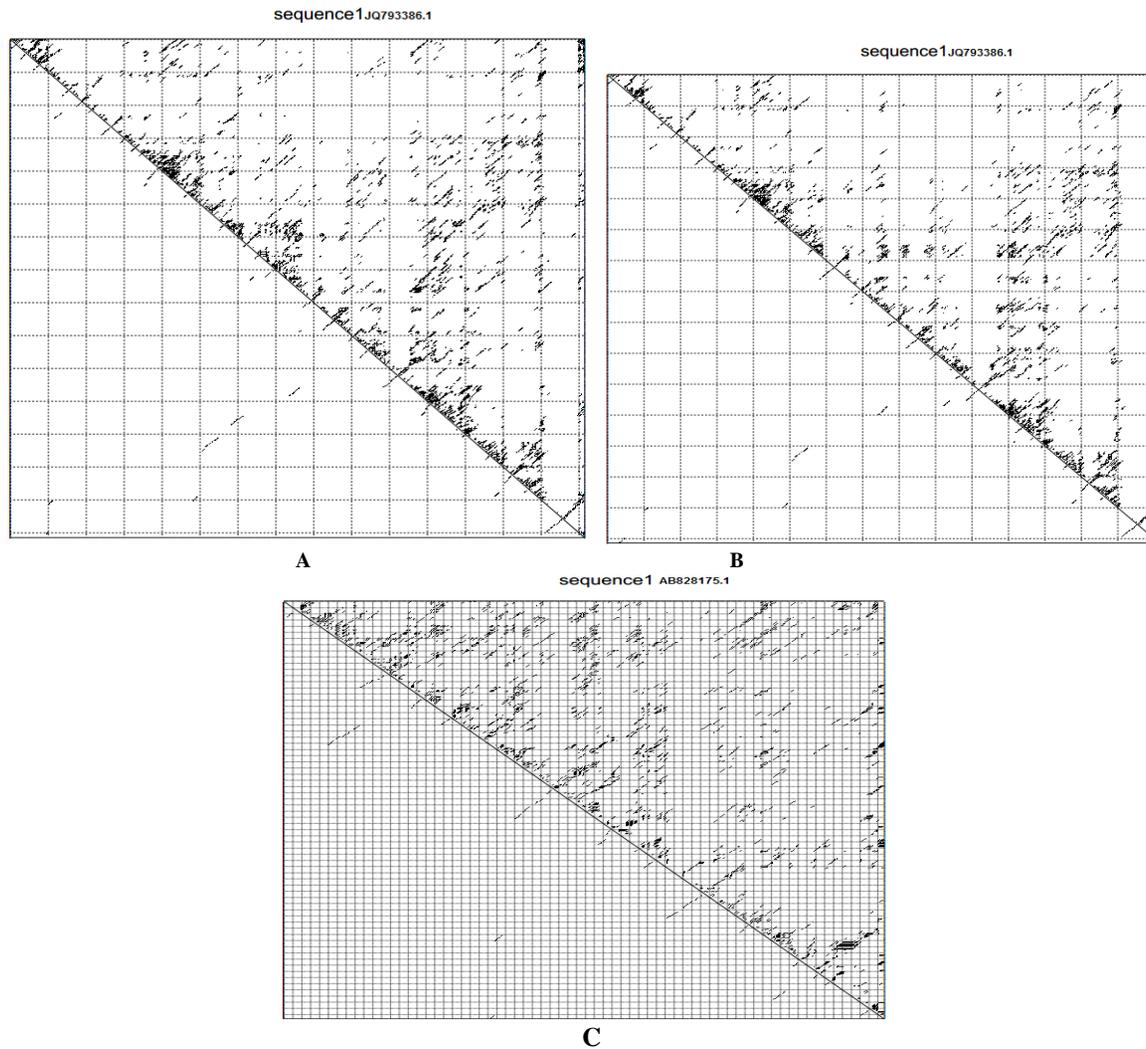


Fig. 8a-c: Screenshots of mountain plot of secondary structure of rRNA in uncultured *Paenibacillus* clone SGR-10, GenBank: JQ793386.1 (A), clone SGR 280, GenBank: JQ793582.1 (B) and clone T-168, GenBank: AB828175(C). There was a variation between uncultured *Paenibacillus* clone T-168, clone SGR10, and clone SGR280. However, there was a high homology between both clone SGR10 and clone SGR280.

DISCUSSION

In this study were discussed the 16S rRNA gene for analysis of the phylogenetic parameters in Uncultured *Paenibacillus* species clone T-168 that we sequenced the 16S rRNA and deposited in GenBank. Usually the variation in 16S rRNA genes in Bacteria is low (Gutler and Stanisich 1996) but our results showed the rate of variation was high between sequences of 16S rRNA genes in uncultured *Paenibacillus* clone T-168 and other species like, uncultured *Paenibacillus* clone SGR-10 and uncultured *Paenibacillus* clone SGR280. In the GenBank, 16S rRNA genes with partial and complete sequences in some species of Bacteria are reported, especially uncultured *Paenibacillus* sp. According to these, we randomly selected two species of uncultured *Paenibacillus*. Since the variation of 16S rRNA genes between species of Bacteria is very low, we selected two species for comparing with uncultured *Paenibacillus* clone T-168. RNA fold web server did analysis between sequences and secondary RNA structures were reported. The RNA fold web server usually utilizes a minimum free energy for evaluation of the secondary structure of the RNA (Szymanski *et al.* 2000; Dynamic *et al.* 1999; Klein and Eddy, 2003). Figures 5a-c, 6a-c, 7a-c and 8a-c show a screenshot of the “Fold RNA Single Strand” input form from the JAVA GUI. This program predicts the lowest free energy structure for an input sequence and a set of low free energy structures called suboptimal structures. The program determined the default output (CT) file and the default parameters for determining suboptimal structures. Saved files can be written to accelerate the prediction of an alternative set of suboptimal structures with a subsequent calculation. This view of the GUI shows the checkbox unchecked and so no saved files will be written. The three remaining parameters control the prediction of suboptimal structures. “Max % Energy Difference” and “Max Number of Structures” place limits on the number of suboptimal structures predicted, with a maximum of the percentage difference in free energy change above the lowest free energy structure or an absolute limit on the number of structures, respectively. Suboptimal structures will be generated until the percent energy difference above the lowest free energy structure is reached, 10% in this case, or 20 structures have been generated (Zuker, 1989). Zero places with no restriction and larger integers become increasingly more stringent in the number of different pairs required between structures. In this studies we discussed the three species of *Paenibacillus* by RNA structure prediction. The results showed that the percent of energy structure between three species was different. The color of nucleotides indicates the rate of variation between species. The optimal secondary structure in dot-bracket notation with a minimum free energy is given in Figures 5a-c, 6a-c, 7a-c and 8a-c. According to these results, the screenshot of RNA secondary structure based on base pairing probabilities at the interactive drawing of the MFE and secondary structure in uncultured *Paenibacillus* sp. Clone T-168, clone SGR280 and clone SGR 10. For unpaired regions, the color denotes the probabilities of being unpaired that were different between three species. There were an existing result, because almost the screenshot of two sequences (*Paenibacillus* clone SGR280 and SGR10) were the same but there was a high variation with sequences of *Paenibacillus* clone T-168. The variations include the shape of RNA secondary structure, the value of energy (the rate of minimum and maximum), and different plot sequencing. The results of comparing the sequences of rRNA with DNAMAN software are also presented in Figure 4. That was different (between sequences of Accession number JQ793582.1, JQ793386.1 and AB8281.75). However same homology between both sequence of Accession number JQ793582.1 and JQ793386.1 were observed. According to the above results, there is a

question that why there were variations between sequences of 16S rRNA in Uncultured *Paenibacillus* clones T-168, SGR-280, and SGR-10. There are some answers to this question:

- 1) The sequence of 16S rRNA in clone T-168, was only acquired from the forward primer, but the other sequences cited in this study are almost complete sequences, so after sequencing full length of this rRNA we can discuss about it. Since the homology of sequences was different when compared with DNAMAN, maybe after getting full length the rate of homology will be higher.
- 2) Maybe uncultured *Paenibacillus* clone T-168 was a new species that produced a mutation variation in the genome of the uncultured *Paenibacillus* species. Because usually there are high variation in 16S rRNA genes within and between species, by these results we would say maybe some mutation penetrated in the genome of uncultured *Paenibacillus* clone T-168, especially in the 16S rRNA gene.
- 3) Maybe there are only some variation in 16S rRNA genes, because after determining the sequence of other genes in *Paenubacilus*, the rate of variation reduced; however these probability is very low.

Finally, only after sequencing the full length of the 16S RNA gene in uncultured *Paenibacillus* clone T-168 we can discuss about the rate of variation between 16S rRNA genes in species of *Paenibacillus*. By the way, there were high variation between 16Sr RNA genes in uncultured *Paenibaclus* clone T-168 and other species of *Paenibacillus*. It is better to study other genes and then decide about the rate of homology between uncultured species clone T-168 and other species of *Paenibacillus*.

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